CYTOCHROME OXIDASE AND SUCCINIC DEHYDROGENASE CONTENT OF SQUID (LOLIGO PEALII) NERVOUS TISSUE

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In the course of investigations designed to elucidate the metabolic differences between normal and chromatolytic nervous tissue, the distribution of succinic dehydrogenase and cytochrome oxidase was studied in various parts of the nervous system of the dog. We found that whereas white matter and spinal nerve had measurable quantities of cytochrome oxidase, both of these tissues contained less succinic dehydrogenase than could be detected by the methods used (Cooperstein, Lazarow and Kurfess, 1950; Schneider and Potter, 1943). In contrast, grey matter and spinal ganglia contain measurable quantities of both of these enzymes and the ratio of cytochrome oxidase to succinic dehydrogenase was found to be 3.9 and 3.2 respectively. If this same enzyme ratio were also present in white matter (or in nerve), the succinic dehydrogenase would have been easily detected. Conversely, considering the sensitivity of the succinic dehydrogenase method employed, it is safe to say that the cytochrome oxidase : succinic dehydrogenase ratio is at least 19.0 in white matter and 9.0 in spinal nerve (Cooperstein, 1951).

Since cytochrome oxidase and succinic dehydrogenase are almost invariably present together in tissues, this dissociation of enzyme activity in nerve and white matter warrants further investigation. It therefore seemed advisable to determine the enzyme content in samples of nerve axoplasm which are not contaminated with the myelin sheath. This can be accomplished in the squid (*Loligo pealii*), where the giant axone can be dissected and samples of pure axoplasm can be obtained in amounts sufficient for microanalyses.

Nachmanson *et al.* (Nachmanson and Meyerhof, 1941; Nachmanson and Steinbach, 1942; Nachmanson, Steinbach, Machado and Spiegelman, 1943) previously studied the distribution of a number of enzymes in squid nervous tissue and found that whereas cholinesterase was more concentrated in the nerve sheath, succinic dehydrogenase and cytochrome oxidase were more concentrated in the axoplasm. Since they used macro-analytical methods, combined samples obtained from different axones were required for each determination. Therefore, relatively few determinations were carried out, and some of the activities reported were at the lower limits of the sensitivity of the methods employed. Furthermore, the assay methods used are less dependable than some of the more recently developed techniques (see Discussion). It therefore seemed advisable to repeat this study using micro-methods (Cooperstein, Lazarow and Kurfess, 1950; Cooperstein and Lazarow, 1951).

Experimental

Methods

Dissection: Squid (*Loligo pealii*) were decapitated and the mantle opened along the mid-ventral line. The bilateral nerve trunks originating from each stel-

late ganglion and containing the giant axone were identified. The giant axone was dissected *in situ* and freed of the extraneous connective tissue sheath and of the small axones which surround the giant axone. The axoplasm is surrounded by a single layer of cells, the inner protoplasmic sheath (which corresponds to the vertebrate Schwann cells), and external to this there is a metatropic sheath which is lipoid-containing. This metatropic sheath, which is similar to the vertebrate myelin sheath except that it lies outside the protoplasmic cell layer, is a few microns thick and can be detected in polarized light (Bear, Schmitt and Young, 1937). It blends with the innermost layers of the outer connective tissue sheath which surrounds the axone, and it usually remains attached to the cleaned giant axone. The right cleaned giant axone with surrounding metatropic sheath was removed. A sample of the fibrous connective tissue sheath (plus the many small axones) which surrounds the giant axone was also taken. The left giant axone was similarly dissected, tied at two places (to prevent loss of axoplasm), removed, and placed on a sheet of Parafilm. One of the ligatures was then removed and a sample of axoplasm obtained by squeezing the nerve between two sheets of Parafilm. Pieces of fin nerve, stellate ganglion, and muscle were also taken. The various samples of tissue weighing between 2 and 10 mg. were placed between two folds of a Parafilm sheet and weighed to 1/100 of a milligram on a Roller-Smith Torsion Balance.[1] The tissue was then transferred to a micro conical-tipped glass homogenizer and kept at 0° C. The Parafilm sheets were then re-weighed. One axone yielded sufficient axoplasm for one set of determinations.

Enzyme determinations: In the succinc dehydrogenase assay (Cooperstein, Lazarow and Kurfess, 1950), the tissues were homogenized at 0° C. in a succinate-phosphate solution (0.5 *M* sodium succinate in 0.2 *M* phosphate buffer, pH 7.4) and diluted with the same solution. The results are expressed as "Standard Enzyme Activity," which is calculated as Δ log [ferricytochrome *c*]/minute for a final tissue dilution of 1 : 100.[2]

In the cytochrome oxidase assay (Cooperstein and Lazarow, 1951), the tissues were homogenized at 0° C. and diluted with 0.033 *M* phosphate buffer, pH 7.4. The results are expressed as "Standard Enzyme Activity," which is calculated as Δ log [ferrocytochrome *c*]/min. for a final tissue dilution of 1 : 100.[3] Two determinations were run on each sample of tissue. In the succinic dehydrogenase assay, 10 μl. and 20 μl. of an appropriate dilution were tested; in the case of the cytochrome oxidase, 2 μl. and 4 μl. were used.

[1] The Parafilm is obtainable from the Marathon Corporation, Menasha, Wisconsin. Suitable weighing papers measuring 10 × 20 millimeters, and weighing between 5 and 10 milligrams are made by stretching the Parafilm sheet at right angles to roll. Tissues enclosed between two folds of Parafilm do not dry out. The Roller-Smith Balance has a total capacity of 25 mg.

[2] Example: When 10 μl. of a 1 : 60 dilution of stellate ganglion were added to 290 μl. of a cytochrome *c*-cyanide solution (final dilution = 1 : 1800) and the logarithms of the extinctions at 550 mμ (corrected for the blank) were plotted against time, the Δ log [cytochrome c^{+++}]/minute was found to be 0.37. This gave a

$$\text{standard enzyme activity} = \frac{\Delta\log[\text{cyto. } c^{+++}]/\text{minute} \times \text{tissue dilution} \times 300}{\text{volume of enzyme added for test} \times 100}$$

$$= \frac{.037 \times 60 \times 300}{10 \times 100} = 0.666.$$

[3] Calculated as in footnote 2.

Results

Succinic dehydrogenase: The succinic dehydrogenase contents of the various tissues are shown in Table I. Stellate ganglion was found to have a significantly higher content of this enzyme than muscle. Although the succinic dehydrogenase contents of the giant axone, fin nerve, fibrous sheath plus small nerves, and axoplasm do not differ significantly from each other, they are all less active than muscle.

TABLE I

Distribution of succinic dehydrogenase in squid tissues

Tissue	Final dilution tested	No. determinations	Average standard enzyme activity (1:100 tissue dilution)	σ*	P† with respect to next lower
Stellate ganglion	1:1800	8	0.682	0.081	< .001
Muscle	1:1200	7	0.466	0.051	< .001
Cleaned giant axone	1:900	6	0.360	0.033	>0.1
Fin nerve	1:900	5	0.340	0.072	>0.1
Fibrous sheath + small nerves	1:900	7	0.337	0.063	>0.1
Axoplasm	1:900	7	0.324	0.060	

* Standard deviation $= \sqrt{\frac{\Sigma(\text{deviations from mean})^2}{N}}$.

† P value of $\frac{\text{Difference}}{\sigma\ \text{Difference}}$.

TABLE II

Distribution of cytochrome oxidase in squid tissues

Tissue	Final dilution tested	No. determinations	Average standard enzyme activity (1:100 tissue dilution)	σ*	P† with respect to next lower
Stellate ganglion	1:15,000	8	6.46	0.827	< .001
Muscle	1:12,000	8	4.60	0.615	< .001
Fibrous sheath + small nerves	1:3,000	8	1.23	0.090	>0.1
Axoplasm	1:3,000	8	1.19	0.012	>0.1
Cleaned giant axone	1:3,000	7	1.12	0.210	>0.1
Fin nerve	1:3,000	4	1.08	0.105	

* Standard deviation $= \sqrt{\frac{\Sigma(\text{deviation from mean})^2}{N}}$.

† P value of $\frac{\text{Difference}}{\sigma\ \text{Difference}}$.

Cytochrome oxidase: Table II shows the distribution of cytochrome oxidase. The tissues were found to have the same relative order of activity with respect to cytochrome oxidase as they do with respect to succinic dehydrogenase.

Ratio cytochrome oxidase/succinic dehydrogenase: Comparing the ratios of cytochrome oxidase to succinic dehydrogenase for the various tissues, it was found that those tissues with the highest enzyme activities also have the highest oxidase to dehydrogenase ratio (ganglion, 9.48; muscle, 9.88), whereas those tissues with the lowest enzyme activities also have the lowest oxidase to dehydrogenase ratio (cleaned giant axone 3.12; axoplasm, 3.67; fibrous sheath, plus small nerves, 3.65; fin nerve, 3.48).

Discussion

Our results do not agree quantitatively with those obtained by Nachmanson *et al.* (Nachmanson and Steinbach, 1942; Nachmanson, Steinbach, Machado and Spiegelman, 1943) in that we did not find succinic dehydrogenase or cytochrome oxidase to be concentrated in the axoplasm. (However, these findings do not alter the primary conclusions of these authors in that the majority of the succinic dehydrogenase and the cytochrome oxidase of squid nerve is present in the axoplasm since the major fraction of the giant axone is axoplasm.) We found that the fibrous connective tissue sheath (plus small nerves) surrounding the giant axone contains as much enzyme as does the axoplasm. We did not determine the enzyme contents of the axonal membrane plus metatropic sheath (residual tissue after squeezing out the cleaned axoplasm) because it is difficult to get sufficient material for analysis. For example, a cleaned giant axone which gave 2.07 mg. of axoplasm yielded only 0.25 mg. of residual tissue (after extrusion of axoplasm).

It is possible that part of the apparent enzyme concentration which they reported for axoplasm may have been due to drying. In our studies we have taken precautions to prevent drying by enclosing the pieces of tissue between two sheets of Parafilm (the weight loss under these conditions is less than 0.05 mg. in two hours), and less than 15 minutes elapsed between removal of the axone and its homogenization.

While the discrepancy in the results is not completely explainable, our methods are probably more reliable than those used by Nachmanson *et al.* Since they report that the respiratory rate of these tissues decreases progressively with time after removal from the animal, the assay methods which we have employed offer the advantage that much less time is required for completion.

In the case of the cytochrome oxidase assay, they used p-phenylenediamine as a substrate. This method has certain drawbacks which interfere with its accuracy (Rosenthal, 1937; Stotz and Hastings, 1937), and these errors are magnified at low oxygen uptakes. Another factor to consider is the possible toxic action of some of the end products of p-phenylenediamine oxidation (Rosenthal, 1937). The method which we used for cytochrome oxidase is not subject to these limitations since cytochrome *c* itself is employed as a substrate.

Their succinic dehydrogenase determinations were carried out in two ways. In the first, the oxygen uptake was measured in the presence of succinate and cytochrome *c*, the activity being a measure of the complete succinoxidase system which includes cytochrome oxidase, cytochrome *c*, succinic dehydrogenase, and probably other unknown factors. The second method utilizes ferricyanide as a direct electron acceptor from succinic dehydrogenase without the intervention of the cytochromes. Although Nachmanson *et al.* report good agreement for the rate of utilization of

succinate as determined by these two methods, they made their calculations on the assumption that oxygen is reduced to hydrogen peroxide by the complete succinoxidase system. If, however, one recalculates the data on the basis that water is the end product, and if the oxidation does not proceed further than fumarate or malate, views which are more generally accepted (Hogness, 1939; LuValle and Goddard, 1948; Keilin and Hartree, 1940), their two methods do not agree and one reaches the conclusion that the rate of oxidation of succinate by the complete succinoxidase system is twice as rapid as its rate of oxidation by one of the components of the succinoxidase system, *i.e.,* succinic dehydrogenase. This is an obvious impossibility, since the rate of oxidation by the complete system can be no faster than the turnover rate of its slowest component. Thus, the turnover rate of the dehydrogenase as measured in the ferricyanide test must be considerably lower than in the natural succinoxidase system. Similar inhibition by another artificial carrier (methylene blue) used in a test for succinic dehydrogenase has also been observed (Potter, 1941; Cooperstein, Lazarow and Kurfess, 1950).[4] The method which we used for the determination of succinic dehydrogenase has the advantage over the ferricyanide test in that the natural substrate, *i.e.,* oxidized cytochrome *c* is used. The advantages of this assay method over that using the complete succinoxidase system as a test for succinic dehydrogenase have been previously discussed.

The enzyme dissociation previously observed in the white matter and spinal nerve of the dog has not been found in squid nerve or samples of squid axoplasm. In fact, there is a lower ratio of cytochrome oxidase : succinic dehydrogenase in squid axoplasm and sheaths than there is in squid stellate ganglion or muscle. Therefore, the low succinic dehydrogenase content observed in dog nerve is not a general phenomenon. It is possible that the large amount of myelin present in this species somehow interferes with the determination of succinic dehydrogenase. However, since the addition of nerve or white matter to other tissues does not result in any inhibition of their succinic dehydrogenase activity, this explanation would not appear to account for the results.

Summary

1. The cytochrome oxidase and succinic dehydrogenase contents of various squid tissues, including giant axone and samples of axoplasm, have been determined and compared with previous findings in dogs. In the case of both enzymes studied, the stellate ganglion of the squid is approximately 1.5 times as active as squid muscle. Squid ganglion has approximately six times as much cytochrome oxidase as the other nervous tissues examined, but only about two times as much succinic dehydrogenase. Our findings, in contrast to previously reported results, reveal no evidence of concentration of these enzymes in axoplasm.

2. The markedly low (or absent) succinic dehydrogenase content which was found in dog nerve and white matter was not observed in the case of the squid nerve, axoplasm, or sheaths.

[4] It has been further reported that a ferricyanide concentration of approximately 1/50th that used in the assay method for succinic dehydrogenese causes a 28 per cent inhibition of the oxygen uptake of the complete succinoxidase system (Barron and Singer, 1945). We have found that a concentration of ferricyanide equal to that used in the assay method causes a 91 per cent inhibition of rat brain succinoxidase.

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